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Ectopic Expression of Activated Stat6 Induces the Expression of Th2-Specific Cytokines and Transcription Factors in Developing Th1 Cells

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Summary

Stat6 is critical for IL-4-mediated Th2 cell development, but its molecular mechanism remains unclear. Here we constructed Stat6:ER, a Stat6-estrogen receptor fusion protein that can be activated by 4-hydroxytamoxifen, independently of IL-4 and endogenous Stat6. Retrovirus-mediated introduction of Stat6:ER into developing Th1 cells induced Th2-specific cytokines and suppressed IFNγ production in a 4-HT-dependent manner and in the absence of IL-4. It also induced GATA-3 and c-*maf* expression and downregulated IL-12Rβ2 chain expression. Its decreased ability to induce the Th2 phenotype with progressing Th1 cell commitment correlated with a decreased induction of GATA-3 and c-*maf*. This study indicates that Stat6 functions upstream of GATA-3 and c-Maf to induce Th2 development.

Introduction

$CD4^+$ T helper cells (Th) develop into at least two distinct subsets with different functional capabilities and cytokine profiles (Mosmann et al., 1986; Mosmann and Coffman, 1989). Th1 cells produce interferon (IFN)-γ and lymphotoxin, confer cell-mediated immunity against intracellular pathogens, and mediate delayed-type hypersensitivity (DTH) and organ-specific autoimmune diseases (Mosmann and Coffman, 1989; Abbas et al., 1996; O'Garra, 1998). In contrast, Th2 cells produce IL-4, IL-5, and IL-13, control the eradication of extracellular helminthic pathogens, and are implicated in atopic and allergic manifestations (Romagnani, 1994). The development of these discrete subsets of Th cells is determined by a number of factors, including cytokines, dose and form of antigens, antigen-presenting cells, costimulators, and the genetic background of the responding host (Abbas et al., 1996; Constant and Bottomly, 1997; O'Garra, 1998). Cytokines, such as IL-12 and IL-4, play a dominant role in driving the development of Th1 and Th2 cells, respectively (Swain et al., 1990; Hsieh et al., 1993; Nelms et al., 1999).

Ligand binding to the IL-4 receptor (IL-4R) activates Jak1 and Jak3, leading to recruitment and phosphorylation of Stat6, IRS-1/2, Shc, and SHP-1 (Ryan et al., 1996; Wang et al., 1996; Zamorano and Keegan, 1998). Distinct regions of the IL-4Rα chain are involved in the activation of these pathways, where the Stat6 pathway transfers a differentiation signal (Ryan et al., 1996; Wang et al., 1996), while the IRS-1/2 pathway is a growth regulator (Keegan et al., 1994; Quelle et al., 1995; Lederer et al., 1996; Ryan et al., 1996; Wang et al., 1996). Stat6 is an 848–amino acid protein and shares homologous domains with other Stat proteins, including an N-terminal domain, a DNA-binding domain (DBD), SH3 and SH2 domains, and a C-terminal transactivation domain (TAD) (Quelle et al., 1995; Lu et al., 1997; Moriggl et al., 1997). Upon phosphorylation of its tyrosine residue, Stat6 homodimerizes, translocates into the nucleus, and binds to specific sequences located in the promoters of IL-4-responsive genes (Hou et al., 1994; Quelle et al., 1995). Stat6-deficient mice show defective Th2 responses, indicating that Stat6 is critical for Th2 cytokine induction (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996; Ouyang et al., 1998). However, activation of Stat6 signaling occurs rapidly (Abbas et al., 1996), while the development of Th2 cells occurs over a few days. It remains unclear whether Stat6 is sufficient to induce a Th2 cell phenotype.

The transcription factors GATA-3 and c-Maf are selectively expressed in Th2 but not Th1 cells (Ho et al., 1996; Zhang et al., 1997; Zheng and Flavell, 1997). GATA-3 strongly transactivates the IL-5 promoter and weakly activates the IL-4 promoter (Zhang et al., 1997; Lee et al., 1998; Ranganath et al., 1998; Zhang et al., 1998). Furthermore, its ectopic expression in developing Th1 cells leads to upregulation of IL-4 and IL-5 and downregulation of IFNγ partly by downregulating the IL-12 receptor β2 (IL-12Rβ2) chain (Zheng and Flavell, 1997; Ouyang et al., 1998; Ferber et al., 1999). c-Maf appears to act as a synergistic factor in Th2-specific cytokine production and downregulates IFNγ production in Th cells cultured under nonskewing conditions (Ho et al., 1996, 1998).

Progressive polarization of $CD4^+$ T cells under Th1- or Th2-inducing conditions ultimately leads to the commitment of mutually exclusive Th phenotypes (Murphy et al., 1996; Nakamura et al., 1997), as observed after chronic antigenic stimulation such as in parasitic diseases or allergic manifestations (Romagnani, 1994). The molecular basis for the commitment of Th phenotypes can be explained, in part, by specific loss of cytokine receptors such as the IL-12Rβ2 chain, which is lost in Th2 cells but maintained in Th1 cells (Rogge et al., 1997; Szabo et al., 1997). Furthermore, IL-4 upregulates the IL-4Rα chain (Kotanides and Reich, 1996) and downregulates the IL-12Rβ2 chain (Szabo et al., 1997), whereas IFNγ upregulates the IL-12Rβ2 chain (Szabo et al., 1997). Moreover, IL-4R-mediated activation of Stat6 and IRS-2 was shown to be blocked in Th1 cells (Huang et al., 1997; Kubo et al., 1997). Undoubtedly, additional molecular events, including the induction of Th-type specific transcription factors such as GATA-3 and c-Maf and the chromatin remodeling of cytokine genes, may also be involved in the commitment of cells toward a Th1 or Th2 phenotype (Ho et al., 1996; Zheng and Flavell, 1997; Agarwal and Rao, 1998a; Murphy et al., 1999).

We have previously shown that 4-hydroxytamoxifen (4-HT)-mediated activation of a Stat6-estrogen receptor

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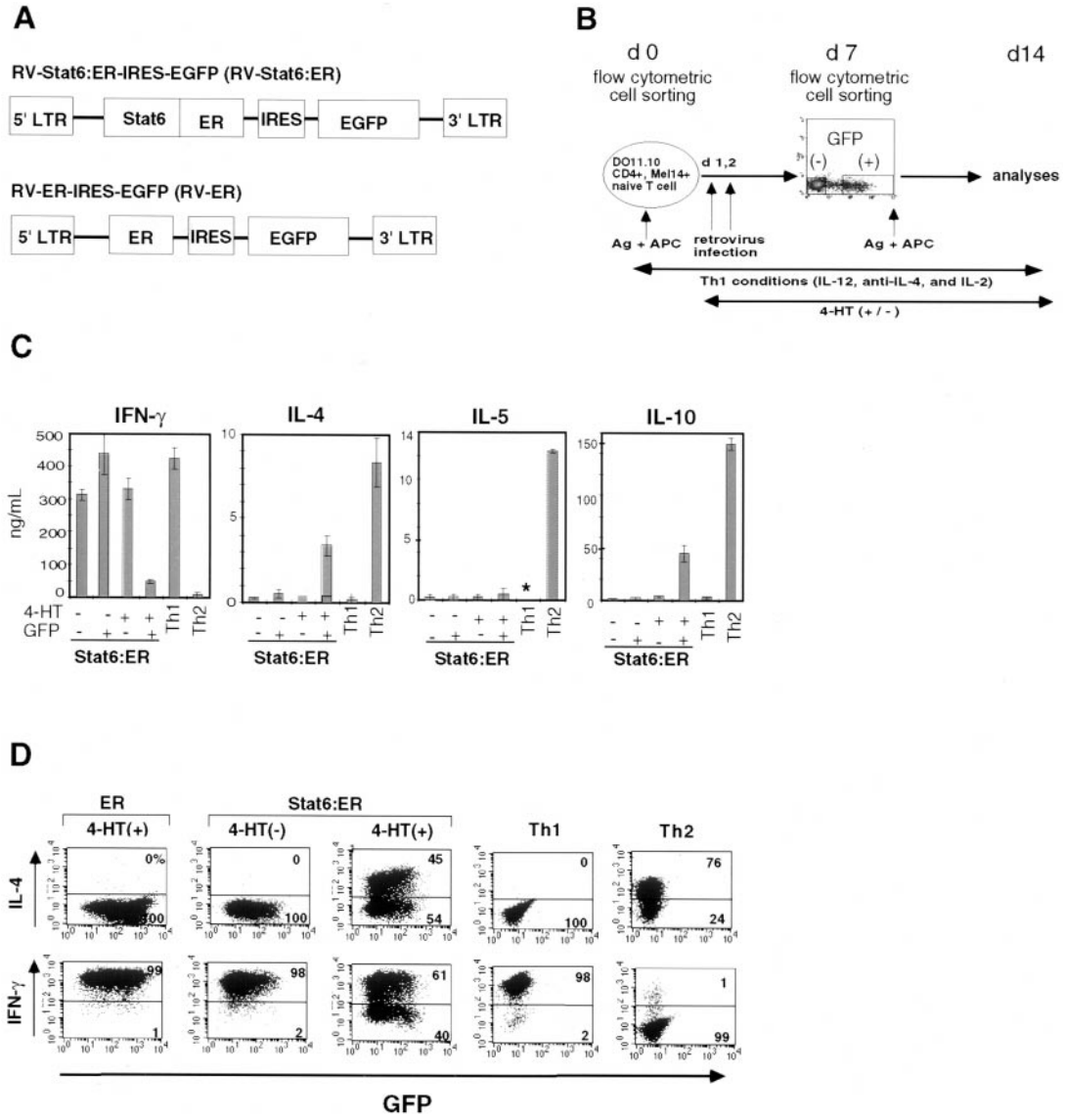

Figure 1. 4-HT-Mediated Activation of Stat6:ER in Developing Th1 Cells Induces Th2 Cytokine Expression and Suppresses IFNγ Expression

(A) Retroviral vectors containing Stat6:ER, ER, and EGFP. RV-Stat6:ER-IRES-EGFP contains a Stat6:ER cDNA, IRES, and EGFP cDNA between the long terminal repeats of the murine stem cell virus (MSCV). RV-ER-IRES-EGFP was used as a control.

(B) Retrovirus infection and analyses. Naive $CD4^+$ T cells from DO11.10 TCR$\alpha\beta$ transgenic mice were infected with retroviruses on days 1 and 2 after activation and cultured under the Th1 condition in the presence (0.3 μM) or absence of 4-HT. GFP-positive and -negative populations were isolated by FCM on day 7 and analyzed for cytokine profiles on days 7 and 14.

(C) Cytokine ELISA of developing Th1 cells infected with RV-Stat6:ER-IRES-EGFP. GFP-positive and -negative cells were harvested on day 7 from cultures in the presence or absence of 4-HT and restimulated at 5×10^5 cells/ml with antigen OVA and APCs for 48 hr. Uninfected Th1 and Th2 cells were analyzed simultaneously. Similar results were obtained on day 14 or by stimulation with PMA and ionomycin. The lower limits of detection were 2.5 ng/ml for IFNγ, 0.4 ng/ml for IL-4, 0.4 ng/ml for IL-5, and 1 ng/ml for IL-10. The asterisks indicate levels lower than the detection limits.

(D) Expression of IL-4 and IFNγ in T cells infected with ER- (control) or Stat6:ER-containing retroviruses. The sorted GFP-positive cells were cultured as in (C), restimulated with PMA and ionomycin for 6 hr, and analyzed for cytokine expression by FCM. Similar results were obtained in four independent experiments.

(ER) fusion protein (Stat6:ER), supposedly by inducing its dimerization and nuclear translocation, mimicked the functional consequences of IL-4-mediated Stat6 tyrosine phosphorylation (Kamogawa et al., 1998). The expression of CD23 was upregulated by activated Stat6:ER, independently of IL-4, in a B lymphoma cell line, M12 (Kamogawa et al., 1998). In the present study, we introduced the Stat6:ER fusion protein into developing Th1 cells by a retroviral vector. Activation of Stat6:ER by 4-HT was sufficient for the induction of Th2-specific cytokines in developing Th1 cells. Moreover, the ability of Stat6:ER to induce a Th2 phenotype correlated with the induction of GATA-3 and c-*maf* mRNA expression.

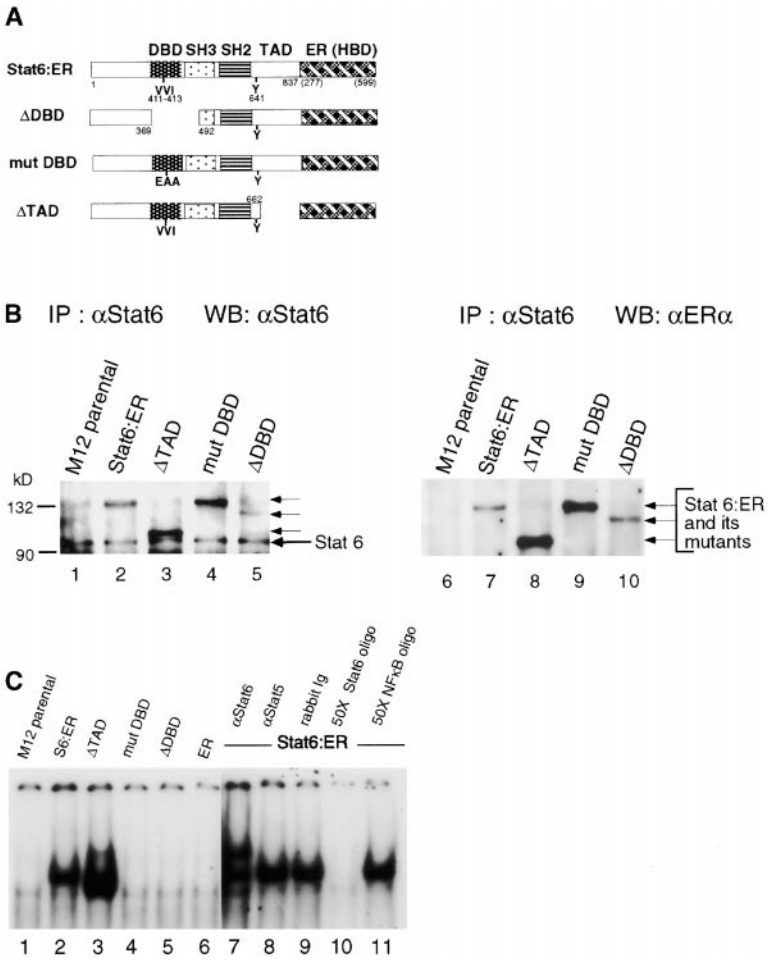

Figure 2. Structures, Expression, and DNA-Binding Activity of Stat6:ER and Its Mutants in M12 Cells

(A) Structures of the Stat6:ER fusion protein and its mutants. Stat6:ER contains a full-length mouse Stat6 and an HBD of ER. ΔDBD contains a deletion of DBD of Stat6. Mut DBD contains a substitution of amino acids at positions from 411 to 413 within DBD. ΔTAD contains a deletion of TAD.

(B) Retrovirus-mediated expression of Stat6:ER or its mutants in M12 cells. M12 cells infected with retroviruses containing Stat6:ER or its mutants were analyzed. Cell lysates were immunoprecipitated with anti-Stat6 antibody, and immunoblotted with anti-Stat6 (lanes 1–5) or anti-ERα antibody (lanes 6–10). Parental M12 cells were also examined (lanes 1 and 6).

(C) EMSA using nuclear extracts from the same cell lines as in (B). The cell lines were cultured in the presence of 1 μM 4-HT for 12 hr. The binding to a probe containing a Stat6-consensus sequence from the mouse IL-4 promoter was examined. The nuclear extracts were preincubated with antibodies (lanes 7–9) or a 50-fold molar excess of unlabeled oligonucleotides (lanes 10 and 11).

Results

Activation of Retrovirally Infected Stat6:ER Induces the Expression of Th2 Cytokines and Suppresses the Expression of IFNγ in Developing Th1 Cells

To directly analyze the role of Stat6 in Th2 development, we constructed Stat6:ER, a conditionally active form of Stat6 that encodes a fusion protein of full-length Stat6 and the hormone-binding domain (HBD) of the estrogen receptor α that is activated specifically by an estrogen analog, 4-HT (Kamogawa et al., 1998). Purified $CD4^+$ Mel-14^{high} naive T cells from DO11.10 TCRαβ transgenic mice were infected by a retroviral vector RV-Stat6:ER-IRES-EGFP (RV-Stat6:ER) that contains a bicistronic element coexpressing Stat6:ER and EGFP (Figure 1A). RV-ER-IRES-EGFP (RV-ER) was used as a control vector (Figure 1A). Cells were activated with antigen and antigen-presenting cells (APCs) and cultured under Th1-inducing conditions (IL-12 plus anti-IL-4) in the presence or absence of 4-HT (Figure 1B). On day 7, the GFP-positive and -negative cells were isolated by flow cytometry (FCM), expanded, and on day 14 analyzed for cytokine production upon stimulation. GFP-positive cells purified from RV-Stat6:ER-infected developing Th1 cells (Stat6:ER Th1 cells) cultured in the presence of 4-HT showed a markedly reduced level of IFNγ and elevated levels of IL-4, IL-5, and IL-10 as compared with control uninfected Th1 cells (Figure 1C). In contrast, GFP-negative cells, as well as Stat6:ER Th1 cells cultured in the absence of 4-HT, were similar to the control uninfected Th1 cells (Figure 1C). Although IL-5 induction by Stat6:ER was not prominent on day 7 (Figure 1C), it became more prominent on day 14 (data not shown). The induction of IL-4 and downregulation of IFNγ in 4-HT-activated Stat6:ER Th1 cells was further confirmed by intracellular cytokine analysis (Figure 1D). After stimulation with PMA and ionomycin on day 14, 45% of Stat6:ER Th1 cells expressed IL-4, while 40% produced markedly reduced levels of IFNγ, only in the presence of 4-HT (Figure 1D), demonstrating the 4-HT-dependent effects of Stat6:ER. In contrast, the sorted GFP-positive cells from RV-ER-infected cells (ER Th1 cells) showed cytokine patterns comparable to control Th1 cells, irrespective of the addition of 4-HT (Figure 1D). Furthermore, cell populations expressing IL-5, IL-6, and IL-10 were increased in the Stat6:ER Th1 cells in a 4-HT-dependent manner (data not shown). The data indicate that Stat6 activation is sufficient for the induction of Th2 cytokines and suppression of IFNγ production in developing Th1 cells.

DNA Binding and Transactivation Domains of Stat6 Are Required for 4-HT-Dependent Transactivation

Stat6 contains several functional domains including DNA-binding (DBD), SH3, SH2, and transactivation

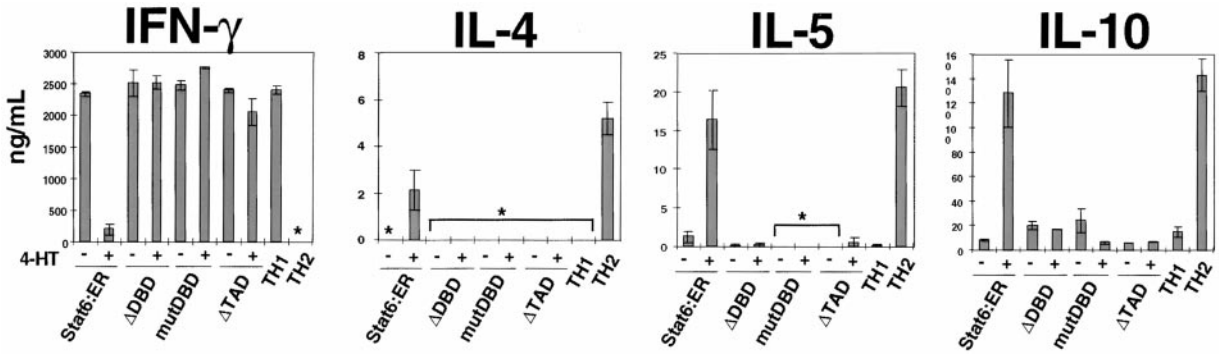

Figure 3. Both the DBD and TAD of Stat6 Are Required for the 4-HT-Mediated Induction of Th2 Cytokine Expression in Developing Th1 Cells

Naive T cells from DO11.10 TCRαβ-transgenic mice were activated as in Figure 1 and infected with retroviruses containing wild-type and mutant Stat6:ERs. GFP-positive cells were restimulated with OVA and APCs on day 14 and analyzed as in Figure 1C. Similar results were obtained by stimulation with PMA and ionomycin.

(TAD) domains (Mikita et al., 1996; Moriggl et al., 1997) (Figure 2A). Three amino acids VVI at positions 411–413 within the DBD are critical for binding to the consensus sequence (Mikita et al., 1996), and the C-terminal TAD is required for its transactivation function (Moriggl et al., 1997).

To determine whether the functional domains of Stat6 are required for the induction of Th2 cell differentiation and to ensure that Stat6:ER was functioning in a Stat6-specific manner, we generated retroviruses containing STAT6:ERs with different mutations, including (1) a deletion of the DBD (ΔDBD), (2) a substitution of three critical amino acids within the DBD (mut DBD), and (3) a deletion of the TAD (ΔTAD) (Figure 2A). Infected M12 cells showed expression of the four Stat6:ERs with predicted sizes: wild-type Stat6:ER and mut DBD, 129 kDa; ΔDBD, 116 kDa; ΔTAD, 110 kDa; and endogenous Stat6, 94 kDa (Figure 2B). We have previously shown by EMSA that the DNA binding of Stat6:ER is induced by 4-HT but not IL-4, while the binding of endogenous Stat6 is induced only by IL-4 (Kamogawa et al., 1998). Among the above four constructs, only the wild-type Stat6:ER and the ΔTAD mutant showed binding activity to the Stat6-consensus element of the mouse IL-4 promoter (Figure 2C, lanes 2 and 3). The binding specificity was confirmed by antibody supershift (lanes 7–9) and competition with unlabeled oligonucleotides (lanes 10 and 11). Furthermore, upregulation of CD23 was induced only by the wild-type Stat6:ER (Kamogawa et al., 1998) but not the three mutants, while all four M12 cell lines upregulated CD23 in response to IL-4 (data not shown). These data indicate the requirement for the same functional domains as endogenous Stat6 for DNA binding and upregulation of CD23 in M12 cells (Mikita et al., 1996).

To examine whether these functional domains of Stat6 were required for the Th2 cytokine production in developing Th1 cells, naive $CD4^+$ T cells infected with retroviral vectors containing wild-type or mutant forms of Stat6:ER were analyzed as described in Figure 1. Only the wild-type Stat6:ER Th1 cells showed a 4-HT-dependent decrease in IFNγ production and increase in IL-4, IL-5, and IL-10 production. In contrast, all three mutant Stat6:ER Th1 cells showed a Th1 cytokine profile, irrespective of the addition of 4-HT (Figure 3). Compatible results were obtained by FCM analysis of intracellular cytokine production (data not shown). These data suggest that both the DBD and TAD of Stat6 are crucial for Stat6:ER-mediated Th2 development.

Activation of Stat6:ER Induces Th2-Specific Cytokine Expression and Suppresses IFNγ in the Complete Absence of IL-4

To address whether the Th2 phenotype induction is a direct effect of Stat6:ER or mediated by a paracrine loop of IL-4, we performed parallel experiments using naive $CD4^+$ T cells (IL-$4^{-/-}$ DO11.10) and APCs (IL-$4^{-/-}$) from IL-4-deficient mice (Kuhn et al., 1991). Stat6:ER Th1 cells from IL-$4^{-/-}$ DO11.10 mice showed 4-HT-dependent increases in IL-5 and IL-10 production and a marked decrease in IFNγ production, in contrast to the control Th1 cells on day 7 (Figure 4A). The same and more accentuated pattern of cytokine production was observed at day 14, while RV-mut DBD-infected developing Th1 cells (mut DBD Th1 cells) and control Th1 cells showed a Th1 pattern (Figure 4B).

Stat6:ER Th1 cells from DO11.10 mice showed a marked increase in IL-4, IL-5, IL-10, IL-13, and IL-6 mRNAs and a decrease in IFNγ and IL-2 mRNAs in a 4-HT-dependent manner, as measured by ribonuclease (RNase) protection (Figure 4C, lanes 3 and 4). A similar change, except for IL-4 mRNA, was observed in IL-$4^{-/-}$ DO11.10 cells (Figure 4C, lanes 5 and 6). Thus, the induction of Th2-specific cytokines and the downregulation of the Th1 phenotype by activated Stat6:ER was independent of IL-4.

Activation of Stat6:ER in Developing Th1 Cells Inhibits IL-12Rβ2 Expression

To test whether Stat6:ER inhibited IFNγ production by downregulating the IL-12Rβ2 chain, we analyzed IL-12 receptor mRNA expression by RNase protection. Uninfected Th1 cells showed a marked increase in the expression of IL-12Rβ2 mRNA, while IL-12Rβ2 mRNA was not detected in developing Th2 cells (Figure 5A, left panel). Stat6:ER Th1 cells preferentially showed decreased expression of IL-12Rβ2 mRNA in a 4-HT-dependent manner. In contrast, mut DBD Th1 cells were comparable to uninfected Th1 cells. The expression of IL-12Rβ2 mRNA was also suppressed by Stat6:ER in IL-$4^{-/-}$ DO11.10

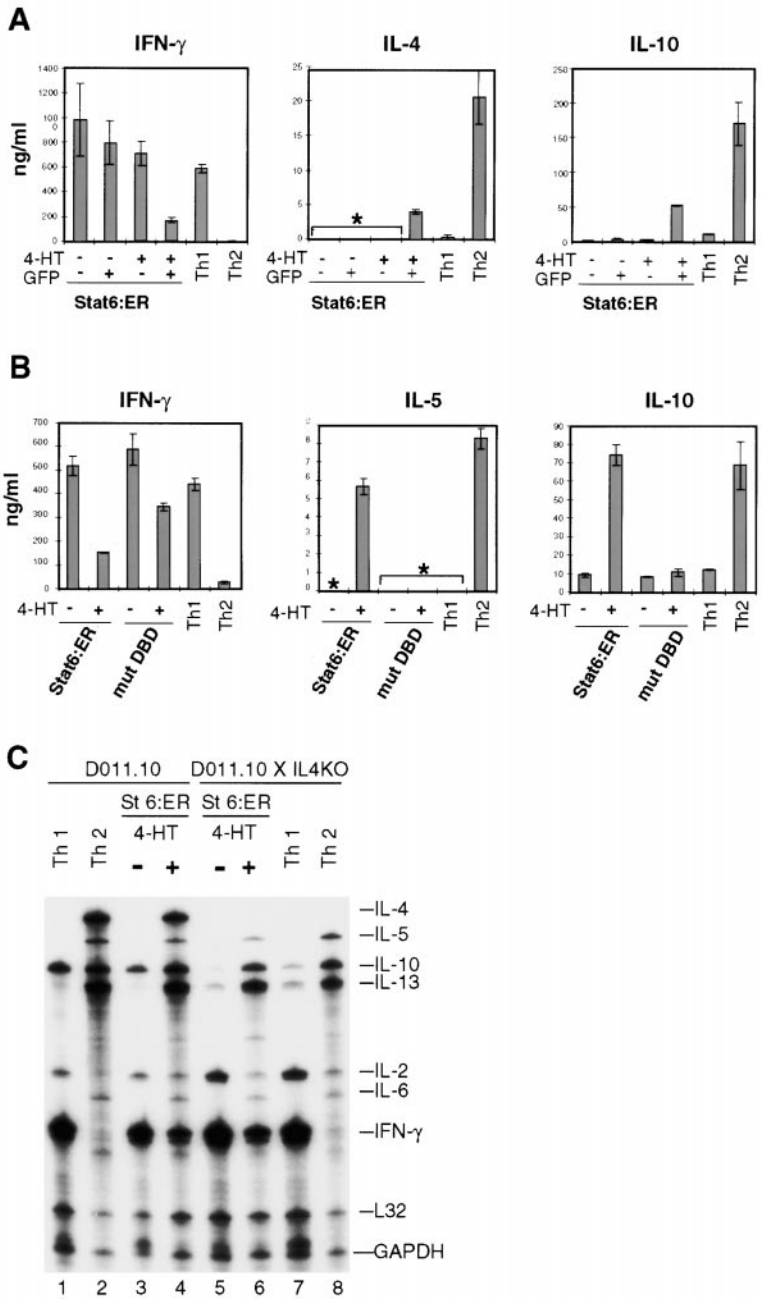

Figure 4. Stat6:ER Induces Th2 Cytokine Expression and Suppresses IFNγ in the Absence of IL-4

IL-4$^{-/-}$ DO11.10 Stat6:ER Th1 cells or mut DBD Th1 cells were analyzed on day 7 (A) and day 14 (B), as in Figures 1C and 3. Control uninfected IL-4$^{-/-}$ DO11.10 T cells were cultured in the Th1 or Th2 culture conditions. Similar results were obtained by stimulation with PMA and ionomycin. (C) RNase protection assay of cytokine mRNAs. Stat6:ER Th1 cells from DO11.10 (lanes 3 and 4) and IL-4$^{-/-}$ DO11.10 mice (lanes 5 and 6) were stimulated with PMA and ionomycin for 6 hr on day 14. Uninfected Th1 and Th2 cells were analyzed simultaneously (lanes 1, 2, 7, and 8).

Th1 cells, although to a lesser extent (Figure 5A, right panel), suggesting that Stat6:ER downregulates IL-12Rβ2 mRNA expression via IL-4-dependent and independent mechanisms.

Activation of Stat6:ER in Developing Th1 Cells Induces the Expression of the Th2-Specific Transcription Factors GATA-3 and c-*maf*

Since GATA-3 (Zheng and Flavell, 1997; Ouyang et al., 1998; Ranganath et al., 1998; Zhang et al., 1998; Ferber et al., 1999) and c-Maf (Ho et al., 1996, 1998) play important roles in Th2 cell development, we examined the effect of Stat6:ER on GATA-3 and c-*maf* mRNA expression by RNase protection. Stat6:ER Th1 cells expressed significant amounts of both GATA-3 and c-*maf* mRNAs in a 4-HT-dependent manner (Figure 5B, lanes 3 and 4), while no significant expression was observed in mut DBD Th1 cells cultured with 4-HT (Figure 5B, lanes 5 and 6). Parallel experiments with IL-4$^{-/-}$ DO11.10 T cells showed that GATA-3 and c-*maf* mRNA expression was also induced in the absence of IL-4 (Figure 5B, lanes 7 and 8), indicating that these genes are downstream targets of Stat6.

Induction of Th2-Specific Cytokines and Inhibition of IFNγ by Activated Stat6:ER Is Limited to Early Stages of Th1 Cell Development

Commitment of Th1 and Th2 cells require multiple rounds of stimulation with antigen and APCs in the presence of IL-12 and IL-4, respectively (Murphy et al., 1996).

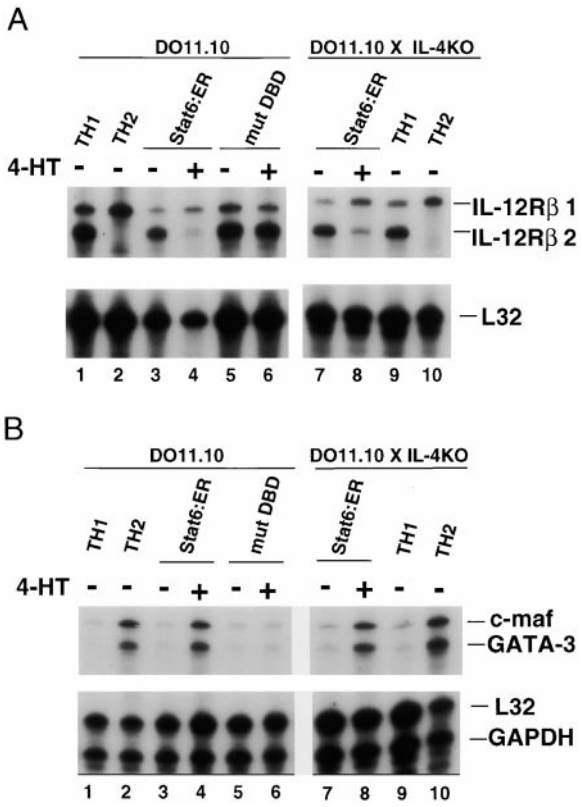

Figure 5. Stat6:ER Induces GATA-3 and c-*maf* mRNA, While Suppressing the Expression of IL-12Rβ2 mRNA in Developing Th1 Cells

(A) RNase protection assay of IL-12Rβ subunit mRNAs. Stat6:ER and mut DBD Th1 cells from DO11.10 (lanes 3–6) and IL-4$^{-/-}$ DO11.10 (lanes 7–8) mice were cultured as in Figure 3. One microgram of total RNA was analyzed for IL-12Rβ1 and 2 subunits and L32 (control housekeeping gene). Uninfected Th1 and Th2 cells were included as in Figure 3 (lanes 1, 2, 9, and 10).

(B) RNase protection assay of GATA-3 and c-*maf* mRNAs. One microgram of total RNA as in (A) was analyzed for GATA-3 and c-*maf* as well as L32 and GAPDH.

Such polarized Th1 cells lose their responsiveness to IL-4 via mechanisms such as selective blocking of Stat6 and IRS-2 pathways (Huang and Paul, 1998). To address whether the block in Stat6 activation is the major mechanism for Th1 commitment, Stat6:ER was activated by 4-HT at various time points during Th1 cell development (Figure 6A). After 2 weeks of treatment with 4-HT, intracellular cytokine profiles were analyzed. The control Stat6:ER Th1 cells, in the absence of 4-HT produced no IL-4, while 98% of them expressed IFNγ (Figure 6A). Early activation of Stat6:ER by addition of 4-HT on days 1–14 (Figure 6A) resulted in a marked increase in IL-4-producing cells (from 0% to 43%) and a significant decrease in IFNγ-producing cells (from 98% to 71%). A 1 week delay in the addition of 4-HT resulted in a lower level of induction of IL-4-producing cells (20%) and a decreased level of suppression of IFNγ-producing cells (93%). Moreover, a 2 or 3 week delay in the addition of 4-HT resulted in substantially lower percentages of IL-4-producing cells (4% and 2%, respectively) and a minimal decrease in IFN-producing cells (93% and 97%, respectively). Similar but more profound results were obtained in a committed Th1 clone HDK1 (Figure 6A, right) in which activation of Stat6:ER showed no differences in IFNγ and no production of IL-4 from uninfected or Stat6:ER HDK1 cells cultured without 4-HT (data not shown). These results suggest that even the forced activation of Stat6:ER cannot convert the phenotype of long-term polarized Th1 cells.

With activation by 4-HT from day 1, the induction of GATA-3 and c-*maf* mRNAs was observed in Stat6:ER Th1 cells (Figure 6B, lane 3) but not in ER Th1 cells (Figure 6B, lane 7). The induction levels of the mRNAs decreased gradually when the start of the addition of 4-HT was delayed (Figure 6B, lanes 4–6). However, if 4-HT was added from as late as day 21, little or no GATA-3 or c-*maf* mRNA expression was observed (Figure 6B, lane 6), which correlated with the levels of Th2 phenotype induction (Figure 6A). Moreover, Stat6:ER activation resulted in no induction of GATA-3 and c-*maf* mRNAs in a committed Th1 clone HDK1 (Figure 6B, lanes 11 and 12), compatible with the inability of Stat6:ER to induce the Th2 phenotype.

Growth Promotion of Stat6:ER-Activated Developing Th1 Cells Is Lost upon Commitment

Although Stat6 and IRS-2 signals are activated by distinct regions of the IL-4 receptor (Ryan et al., 1996), Stat6-deficient lymphocytes have been shown to be defective in their proliferative responses to IL-4 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). To investigate whether activated Stat6 can enhance the proliferation of developing Th1 cells, we examined the growth of Stat6:ER Th1 cells treated with different concentrations of 4-HT by [^{3}H]thymidine incorporation (Figure 7A). The proliferation of Stat6:ER Th1 cells was enhanced in a dose-dependent manner on days 10 and 31 after polarization (Figure 7A), in contrast with that of the ER Th1 or uninfected Th1 cells (the effective concentration of 4-HT was between 0.08 and 2 μM, and the toxic concentration was greater than 5 μM). A marked enhancement of proliferation was also observed in the RV-Stat6:ER-infected Th2 clone D10 (Figure 7B, upper right) but not in the RV-Stat6:ER-infected Th1 clone HDK1 (Figure 7B, upper left). The growth enhancement was Stat6-dependent, since none of the clones expressing mutant Stat6:ERs induced a growth enhancement in response to 4-HT (Figure 7B, upper right), while all of the clones responded equally to IL-2 (Figure 7B, lower panels). These data indicate that Stat6 induced growth promotion in a Th2 clone and developing Th1 cells, but that this effect is lost in a committed Th1 clone.

Discussion

Gene targeting studies have revealed that Stat6 is critical for Th2 cell development (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996) as well as the induction of GATA-3 (Ouyang et al., 1998). However, it remains unclear how Stat6 activates the cascade of events leading to Th2 cell development. In this study, using 4-HT-mediated activation of Stat6:ER, we showed that Stat6 activation is sufficient to induce the production of Th2 cytokines and the downregulation of IFNγ in developing Th1 cells independently of other IL-4 signals. Furthermore, activated Stat6 led to the downregulation

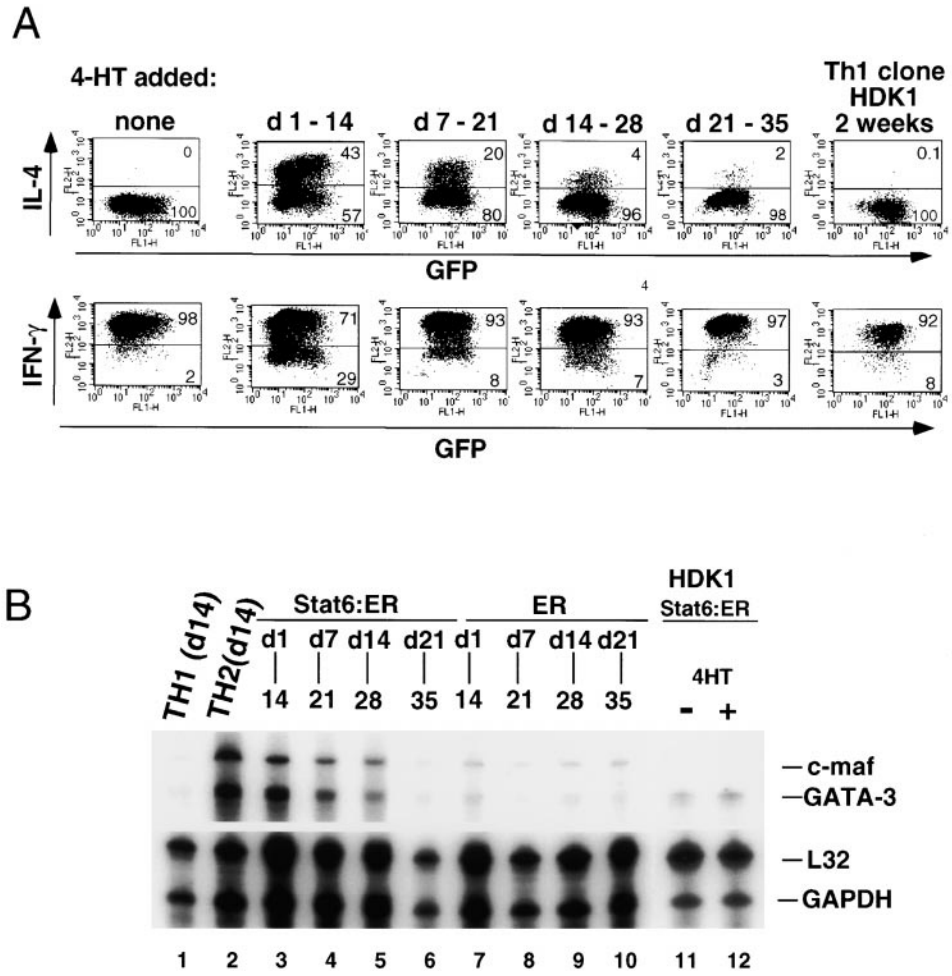

Figure 6. The Induction of Th2 Phenotypes by Stat6:ER Is Limited to Early Stages of Th1 Cell Development

(A) Naive DO11.10 T cells were activated and infected with RV-Stat6:ER, as in Figure 1. 4-HT (0.3 μM) was added for 2 weeks starting from days 1, 7, 14, or 21, and the cells were analyzed for cytokine expression as in Figure 1D. Stat6:ER Th1 cells cultured without 4-HT were also shown (left panels). ER Th1 cells showed the same phenotype as the Th1 control, even with the addition of 4-HT from days 1 to 14 (data not shown). Committed Th1 clone HDK1 infected with RV-Stat6:ER and cultured with 0.3 μM of 4-HT for 2 weeks was analyzed in the same way (right panels).

(B) RNase protection assay of GATA-3 and c-*maf* mRNAs. One microgram of total RNA in the same samples as (A) was analyzed for GATA-3 and c-*maf*, as in Figure 5B. Control uninfected Th1 and Th2 cells cultured for 2 weeks (lanes 1 and 2) as well as RV-Stat6:ER-infected HDK1 cells (lanes 11 and 12) were also examined.

of the IL-12Rβ2 chain as well as the induction of GATA-3 and c-*maf* mRNAs. The ability of Stat6 to induce the Th2 phenotype was restricted to an early stage of Th1 cell development, while no Th2 phenotype was induced by Stat6 activation in committed Th1 cells and a Th1 clone. These results clearly demonstrate that Stat6 functions upstream of GATA-3 and c-Maf as the initiator of the events leading to Th2 development.

In this study, we used a fusion protein of Stat6 to the HBD of ER, which neither binds estradiol nor possesses inherent ligand-dependent transactivation activity but retains responsiveness to 4-HT (Littlewood et al., 1995). We previously showed that activated Stat6:ER upregulated CD23 in M12 cells in a manner similar to endogenous Stat6 activated by IL-4 (Kamogawa et al., 1998), and the binding specificity of Stat6:ER to the Stat6-consensus sequence was confirmed by EMSA (Figure 2C) (Kamogawa et al., 1998). We now demonstrate that wild-type Stat6:ER but not its mutants induced a Th2 phenotype in developing Th1 cells (Figure 3), confirming that the biological functions of Stat6:ER described herein are derived from Stat6 but not the HBD of ER (Pritchard et al., 1995).

Introduction and activation of Stat6:ER in developing Th1 cells allowed us to dissect the sequence of events ensuing from IL-4R activation. The advantage of this system is that Stat6:ER can be introduced into naive $CD4^+$ T cells at one time point and its activation can be delayed until the addition of 4-HT. Using this system, we showed that the activation of Stat6:ER at an early stage during Th1 development led to the induction of IL-4 and IL-5 and downregulation of IFNγ, while its activation at later stages did not (Figure 6A).

The molecular basis for commitment to a Th1 or Th2 phenotype can likely be explained by multiple mechanisms, including (1) differential cytokine signaling, including regulated cytokine receptor expression; (2) differential expression of Th-specific transcription factors;

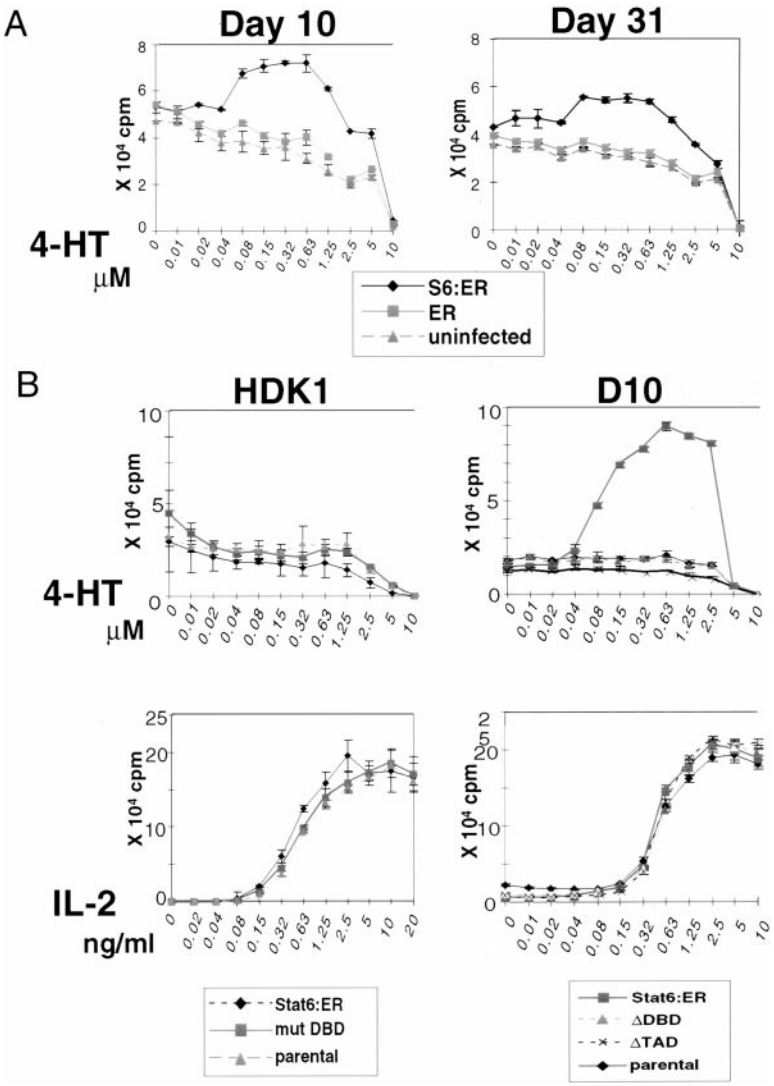

Figure 7. Cell Proliferation Is Specifically Activated by Stat6:ER in Developing Th1 Cells and a Committed Th2 Clone but Not a Committed Th1 Clone

(A) Proliferation assays were performed with Stat6:ER Th1, ER Th1, and uninfected Th1 cells on day 10 and 31 after the initial stimulation. Cells were cultured in Th1 culture conditions in the presence of serially diluted concentrations of 4-HT for 48 hr, and [^{3H}]thymidine was added for the last 6 hr.

(B) Proliferation assays were also performed with HDK1 cells (left) and D10 cells (right), which were infected by retroviruses containing Stat6:ER and its mutants. These cells were incubated with various concentrations of 4-HT in the presence of 1 ng/ml of IL-2 (upper panels) or various concentrations of IL-2 (lower panels) for 48 hr, and [^{3}H]thymidine was added for the last 6 hr.

and (3) differential chromatin remodeling of Th1- and Th2-specific genes. With respect to differential cytokine signaling, commitment to the Th2 phenotype led to a rapid loss of IL-12 signaling (Szabo et al., 1997). This can be achieved by a balance between IL-4-mediated downregulation and IFNγ-mediated upregulation of the IL-12Rβ2 chain (Szabo et al., 1995, 1997; Rogge et al., 1997). Thus, it is possible that activation of Stat6:ER may lead to downregulation of IFNγ production partly by inhibiting IL-12Rβ2 chain expression in developing Th1 cells. In contrast, the irreversible phenotype of committed Th1 cells has been explained by a block of Stat6 activation. Tyrosine phosphorylation of JAK3 and Stat6 was selectively blocked in Th1 cells, despite the expression levels of the IL-4R and Stat6 in Th1 cells being similar to those in Th2 cells (Kubo et al., 1997; Huang and Paul, 1998). In addition, Stat6 may bind to a repressor element in the 3′ untranslated region of the IL-4 gene and release its suppressive effects in Th2 but not Th1 cells (Kubo et al., 1997). However, since we could not induce Th2-specific cytokines in committed Th1 cells by activation of Stat6:ER, it is possible that blocking Stat6 activation may not be the only reason that a committed Th1 phenotype is irreversible; rather, Th2-specific factors other than Stat6 may be lacking in committed Th1 cells.

Commitment to the Th2 lineage is also dictated by the differential expression of GATA-3 and c-Maf, both of which are important for the regulation of Th1- or Th2-specific cytokine genes (Ho et al., 1996; Zhang et al., 1997, 1998; Zheng and Flavell, 1997; Lee et al., 1998; Ouyang et al., 1998; Ranganath et al., 1998; Ferber et al., 1999) (reviewed in O'Garra, 1998). Ectopically expressed GATA-3 in developing Th1 cells induced Th2-specific cytokines and suppressed IFNγ production (Zheng and Flavell, 1997; Ouyang et al., 1998; Ferber et al., 1999). However, the induction of Th2-specific cytokines by ectopically expressed GATA-3 is limited to an early stage of Th1 development but is not observed in polarized Th1 cells or clones (Ouyang et al., 1998). Our study revealed that the ability of Stat6:ER to induce Th2-specific cytokines progressively decreased during differentiation and correlated well with the levels of GATA-3 and c-*maf* mRNA induction (Figure 6B). We confirmed that

Stat6:ER was functional in committed Th1 cells because (1) levels of GFP expression reflecting the bicistronic transcript and thus Stat6:ER expression were not different among Stat6:ER Th1 cells activated at various time points (Figure 6A), (2) 4-HT-dependent binding to a Stat6-consensus sequence probe was still observed by EMSA in nuclear extracts from Stat6:ER Th1 cells obtained on day 28 after polarization but not in extracts from mutant Stat6:ER Th1 cells (data not shown), and (3) 4-HT-dependent growth enhancement was induced by Stat6:ER on day 10 and 31 (Figure 7). The reason for the decreased induction of GATA-3 by Stat6:ER remains unclear, although the autostimulatory induction of endogenous GATA-3 by retrovirally expressed GATA-3 was also lost in committed Th1 cells and a Th1 clone (H. J. L., unpublished data). It is possible that the components required for GATA-3 gene activation may be lost during Th1 development or that chromatin remodeling rendered the GATA-3 gene inaccessible to transcription factors. However, we recently observed that high levels of ectopic expression of GATA-3 in committed Th1 cells and a Th1 clone could induce substantial levels of IL-4 and IL-5 production (H. J. L., unpublished data). Taken together, our studies indicate that GATA-3 and c-Maf are downstream of Stat6 in the sequence of events leading to Th2 cell development and that this pathway is blocked in committed Th1 cells.

It has recently been shown that the development of naive Th cells into Th1 or Th2 cells is associated with differential chromatin remodeling of IFNγ as well as IL-4 and IL-13 genes (Agarwal and Rao, 1998a; Takemoto et al., 1998), which may be accompanied by DNA demethylation and histone acetylation (Agarwal and Rao, 1998a; Bird et al., 1998; Fitzpatrick et al., 1998) and changes in DNase hypersensitivity as in globin gene regulation and erythroid differentiation by GATA-1 (Stamatoyannopoulos et al., 1995). Although the locus control regions and their binding proteins have not been identified, GATA-3 and Stat6 might be strong candidates for long-range chromatin remodeling of the Th2 cytokine gene cluster (Agarwal and Rao, 1998b). During Th1 cell development, chromatin structures of Th2-specific cytokine genes might become more inaccessible to the transcription factors or resistant to chromatin remodeling, reflecting an irreversible Th1 phenotype (Agarwal and Rao, 1998a).

The development of Th1 and Th2 cells has recently been shown to require cell cycle progression (Bird et al., 1998). It has been suggested that during IL-4 signaling the Stat6 pathway mediates differentiation, while the IRS-1/2 and Shc pathways stimulate cell proliferation (Keegan et al., 1994; Quelle et al., 1995; Lederer et al., 1996; Ryan et al., 1996; Wang et al., 1996; Zamorano and Keegan, 1998). However, both T and B lymphocytes from Stat6-deficient mice have been shown to proliferate poorly in response to IL-4 (Kaplan et al., 1996; Shimoda et al., 1996; Takeda et al., 1996). Moreover, lymphocytes from Stat6-deficient mice have reduced IL-4-mediated cdk2 activity and downregulation of $p27^{Kip1}$ expression, leading to defective cell cycle progression (Kaplan et al., 1998). It is still possible that Stat6 may mediate its effects on proliferation indirectly as hypothesized by Keegan and Paul (Ryan et al., 1996; Wang et al., 1996; Zamorano and Keegan, 1998). In support of this, activated Stat6:ER enhanced the proliferation of developing Th1 cells, examined on day 10 and 35 following the initial activation, but not of the committed Th1 clone (Figure 7), suggesting that Stat6-mediated growth enhancement may require other factors existing in a Th2 clone and developing Th1 cells but not in a Th1 clone.

In conclusion, our results revealed that activated Stat6:ER, in the absence of IL-4, transfers signals sufficient for the induction of Th2 cytokines and suppression of IFNγ in developing but not committed Th1 cells. These activities strictly correlated with the induction of GATA-3 and c-*maf* expression, indicating that Stat6 functions upstream of these transcription factors.

Experimental Procedures

Mice, Cytokines, Antibodies, and Cell Lines

DO11.10 TCR$\alpha\beta$ transgenic mice (Murphy et al., 1990) and DO11.10 TCR$\alpha\beta$ transgenic crossed with IL-$4^{-/-}$ mice (Kuhn et al., 1991) were maintained as previously described (Robinson et al., 1997).

Purified recombinant mouse IL-2 and IL-4 (DNAX), IL-12 (PharMingen), and rat anti-mouse IL-4 and anti-IL-12 antibodies (kind gifts of Drs. W. E. Paul and G. Trinchieri, respectively) were used for cell culture. Polyclonal rabbit anti-mouse Stat6 and anti-mouse estrogen receptor-α antibodies (Santa Cruz Biotechnology) were used for immunoprecipitation, immunoblotting, and EMSA. For intracellular staining, phycoerythrin (PE)-labeled anti-mouse IL-4 antibody (PharMingen) and anti-mouse IFNγ antibody (DNAX) were used.

HDK1, D10, and M12 cells were cultured as previously described (Kaye et al., 1983; Cherwinski et al., 1987; Kamogawa et al., 1998). A retrovirus packaging cell line, Phoenix-Eco (Dr. G. Nolan, Stanford) was cultured in DMEM containing 10% FCS in the presence of diphtheria toxin (1 μg/ml) and hygromycin B (375 μg/ml) and used after three days of culture without the drugs.

Preparation of Naive $CD4^+$ T Cells

Splenic naive T cells were prepared as previously described (Ferber et al., 1999). Briefly, $CD4^+$ T cell–rich populations were collected from splenic cells by immunomagnetic negative selection using anti-CD8α, anti-Mac-1, and anti-B220 antibodies using BioMAG Separators (PerSeptive Biosystems). Naive $CD4^+$ T cells were purified by sorting $CD4^+$ Mel-14^{high} populations on a FACStarplus (Becton Dickinson) with a purity of above 99%. They were stimulated with antigen (0.6 μM $OVA_{323–339}$) and 3000 rad irradiated BALB/c mouse splenic cells at a density of naive T cells and splenic APCs of 1.25×10^5 and 2.5×10^6 cells/ml, respectively, in T cell medium containing 10 ng/ml IL-2 (Murphy et al., 1996). For Th1 cultures, 5 ng/ml of IL-12 and 10 μg/ml of anti-IL-4 antibody were added, and, for Th2 cultures, 10 ng/ml of IL-4 and 10 μg/ml of anti-IL-12 antibody were added (Murphy et al., 1996).

Construction of Retroviral Constructs

Enhanced green fluorescent protein (EGFP) encoding vector plasmid pMXI-EGFP was provided by Dr. A. Mui (DNAX). pMXI-EGFP was prepared by insertion to pMX (Dr. T. Kitamura, DNAX) (Onishi et al., 1996) with a BamHI–NotI fragment from the LZRSpBMN-linker-IRES-EGFP (Dr. H. Spits, Netherlands Cancer Center), which contains multi-cloning sites and an internal ribosome entry site (IRES) from encephalomyocarditis virus (EMCV) and an EGFP sequence from pEGFP-1 (Clontech Laboratories). To generate pMX-Stat6:ER-IRES-EGFP (pMX-Stat6:ER), an EcoRI–NotI fragment of pBabe-puro-Stat6:ER-IRES-EGFP (Dr. Y. Kamogawa, DNAX) was introduced into the pMXI-EGFP vector. To generate pMX-ER-IRES-EGFP containing the HBD of the mouse estrogen receptor α (amino acids [aa] 281–599 with an amino acid substitution of Gly to Arg at position 525) (ER), an EcoRI–SalI fragment from pBabe-puro-hbER* (Dr. M. McMahon, DNAX) (Woods et al., 1997) was inserted into pMXI-EGFP. pMX-ΔDBD Stat6:ER-IRES-EGFP was prepared by removing a 363 bp Eco47III–StuI fragment corresponding to aa 370–

491 of Stat6 from pMX-Stat6:ER. pMX-mut DBD Stat6:ER-IRES-EGFP was constructed by PCR-directed mutagenesis of EAA for VVI at positions aa 411–413 of Stat6 (Mikita et al., 1996). pMX-ΔTAD Stat6:ER-IRES-EGFP was prepared by removing a 525 bp fragment corresponding to aa 663–837 of Stat6.

Preparation of Retroviruses and Infection

Phoenix-Eco packaging cell line was transfected with retroviral plasmids using Lipofectamine PLUS (GIBCO–BRL) according to the manufacturer's protocol. Purified naive T cells were activated with antigen and APCs, as described above, and then infected with retrovirus-containing supernatants in the presence of 0.5 μg/ml polybrene at 32°C 1 and 2 days after activation (Figure 1B) (Ferber et al., 1999). They were cultured and expanded under Th1 culture conditions in the presence or absence of 0.3 μM 4-HT (Research Biochemicals Institute) (Figure 1B). GFP-positive and -negative T cells were sorted on day 7 with a purity of above 98%. The frequency of infection was usually between 20% and 30%. The sorted cells were activated weekly and cultured as described above (Figure 1B). M12 cells were infected with retrovirus as described previously (Kamogawa et al., 1998).

Immunoprecipitation and Immunoblotting

Preparation of total cell lysates from M12 cells expressing Stat6:ER and its mutants was performed as previously described (Kamogawa et al., 1998). Detergent-soluble fractions were incubated with 2 μg of anti-Stat6 antibody together with 40 μl of Protein A (Pierce) overnight at 4°C. Immune complexes were separated by SDS-PAGE, transferred to Immobilon-P membrane (Millipore), and detected with anti-ERα- or anti-STAT6 antibody using SuperSignal Substrate detection system (Pierce).

Preparation of Nuclear Extracts and EMSA for Stat6

Nuclear extracts were prepared from 1×10^8 M12 cells as described (Kamogawa et al., 1998). Nuclear extracts (4 μg of protein) were incubated with 1 ng of ^{32}P-labeled probe. Double-stranded oligonucleotide probe (5′-GATCTGATTTCACAGGAAAATT-3′ and 5′-GATCAATTTTCCTGTGAAATCA-3′) contains a core sequence corresponding to the Stat6 responsive element (underlined) of the mouse IL-4 promoter. In supershift assay, nuclear extracts were preincubated with 1 μg of each antibody at room temperature for 15 min. In oligomer competition, a 50-fold molar excess of unlabeled Stat6 or NF-κB oligo nucleotides was added.

Proliferation Assays

Cells were incubated at 1×10^5/round-bottom 96-well plate with various concentrations of 4-HT in Th1 culture medium, as described above, or IL-2 in T cell culture medium. Cells were pulsed with 1 μCi [^{3}H]thymidine for the last 6 hr of a 48 hr culture period (Kaplan et al., 1996).

Flow Cytometric Analyses of Intracellular Cytokine Profiles

For intracellular cytokine staining, cells were resuspended at 1×10^6/ml and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hr. Two hours before cell harvest, brefeldin A (10 μg/ml) was added. Cells were washed, fixed with formaldehyde (final concentration 2%) in PBS for 20 min, and stained with anti-cytokine antibodies as previously described (Murphy et al., 1996).

Immunoassay for Cytokine Production

Cells (5×10^4) were stimulated in 200 μl cultures in 96-well plates with PMA (50 ng/ml) and ionomycin (500 ng/ml) or 1×10^6 3000 rad irradiated splenic cells and 0.6 μM OVA peptide for 48 hr. Supernatants were collected and examined by immunoassay. IL-4, IL-5, IL-10, and IFNγ were detected as described previously (Robinson et al., 1997). Calculated values were expressed as means ± SEM.

RNase Protection Assay

Total RNA was isolated using Qiagen RNeasy system (Qiagen). RNase protection assay was performed with RiboQuant multiprobe kit (PharMingen) following the manufacturer's method using mCK-1 and mCR-3 multi-probe template sets, GAPDH, L32 (PharMingen), GATA-3, and c-*maf* probes (H. J. L., unpublished data). Transcript levels were quantified by autoradiography using fluorescent scanner Storm (Molecular Dynamics).

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References

Abbas, A.K., Murphy, K.M., and Sher, A. (1996). Functional diversity of helper T lymphocytes. Nature *383*, 787–793.

Agarwal, S., and Rao, A. (1998a). Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. Immunity *9*, 765–775.

Agarwal, S., and Rao, A. (1998b). Long-range transcriptional regulation of cytokine gene expression. Curr. Opin. Immunol. *10*, 345–352.

Bird, J.J., Brown, D.R., Mullen, A.C., Mosckowitz, N.H., Mahowald, M.A., Sider, J.R., Gajewski, T.F., Wang, C.R., and Reiner, S.L. (1998). Helper T cell differentiation is controlled by the cell cycle. Immunity *9*, 229–237.

Cherwinski, H.M., Schumacher, J.H., Brown, K.D., and Mosmann, T.R. (1987). Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. *166*, 1229–1244.

Constant, S.L., and Bottomly, K. (1997). Induction of Th1 and Th2 CD4$^+$ T cell responses: the alternative approaches. Annu. Rev. Immunol. *15*, 297–322.

Ferber, I.A., Lee, H.J., Zonin, F., Heath, V., Mui, A., Arai, N., and O'Garra, A. (1999). GATA-3 significantly downregulates IFN-γ production from developing Th1 cells in addition to inducing IL-4 and IL-5 levels. Clin. Immunol. *91*, 134–144.

Fitzpatrick, D.R., Shirley, K.M., McDonald, L.E., Bielefeldt-Ohmann, H., Kay, G.F., and Kelso, A. (1998). Distinct methylation of the interferon gamma (IFN-γ) and interleukin 3 (IL-3) genes in newly activated primary CD8$^+$ T lymphocytes: regional IFN-γ promoter demethylation and mRNA expression are heritable in CD44^{high} CD8$^+$ T cells. J. Exp. Med. *188*, 103–117.

Ho, I.-C., Hodge, M.R., Rooney, J.W., and Glimcher, L.H. (1996). The proto-oncogene c-*maf* is responsible for tissue-specific expression of interleukin-4. Cell *85*, 973–983.

Ho, I.C., Lo, D., and Glimcher, L.H. (1998). c-maf promotes T helper cell type 2 (Th2) and attenuates Th1 differentiation by both interleukin 4-dependent and -independent mechanisms. J. Exp. Med. *188*, 1859–1166.

Hou, J., Schindler, U., Henzel, W.J., Ho, T.C., Brasseur, M., and McKnight, S.L. (1994). An interleukin-4-induced transcription factor: IL-4 Stat. Science *265*, 1701–1706.

Hsieh, C.S., Macatonia, S.E., Tripp, C.S., Wolf, S.F., O'Garra, A., and Murphy, K.M. (1993). Development of T_H1 CD4$^+$ T cells through IL-12 produced by Listeria-induced macrophages. Science *260*, 547–549.

Huang, H., Hu, L.J., Chen, H., Ben, S.S., and Paul, W.E. (1997). IL-4 and IL-13 production in differentiated T helper type 2 cells is not IL-4 dependent. J. Immunol. *159*, 3731–3738.

Huang, H., and Paul, W.E. (1998). Impaired interleukin 4 signaling in T helper type 1 cells. J. Exp. Med. *187*, 1305–1313.

Kamogawa, Y., Lee, H.J., Johnston, J.A., McMahon, M., O'Garra,

A., and Arai, N. (1998). A conditionally active form of STAT6 can mimic certain effects of IL-4. J. Immunol. *161*, 1074–1077.

Kaplan, M., Schindler, U., Smiley, S.T., and Grusby, M.J. (1996). Stat6 is required for mediating responses to IL-4 and for the development of Th2 cells. Immunity *4*, 313–319.

Kaplan, M.H., Daniel, C., Schindler, U., and Grusby, M.J. (1998). Stat proteins control lymphocyte proliferation by regulating $p27^{Kip1}$ expression. Mol. Cell. Biol. *18*, 1996–2003.

Kaye, J., Porcelli, S., Tite, J., Jones, B., and Janeway, C.A. (1983). Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. J. Exp. Med. *158*, 836–856.

Keegan, A.D., Nelms, K., White, M., Wang, L.M., Pierce, J.H., and Paul, W.E. (1994). An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. Cell *76*, 811–820.

Kotanides, H., and Reich, N.C. (1996). Interleukin-4-induced STAT6 recognizes and activates a target site in the promoter of the interleukin-4 receptor gene. J. Biol. Chem. *271*, 25555–25561.

Kubo, M., Ransom, J., Webb, D., Hashimoto, Y., Tada, T., and Nakayama, T. (1997). T-cell subset-specific expression of the IL-4 gene is regulated by a silencer element and STAT6. EMBO J. *16*, 4007–4020.

Kuhn, R., Rajewsly, K., and Muller, W. (1991). Generation and analysis of interleukin-4 deficient mice. Science *254*, 707–710.

Lederer, J.A., Perez, V.L., DesRoches, L., Kim, S.M., Abbas, A.K., and Lichtman, A.H. (1996). Cytokine transcriptional events during helper T cell subset differentiation. J. Exp. Med. *184*, 397–406.

Lee, H.J., O'Garra, A., Arai, K., and Arai, N. (1998). Characterization of cis-regulatory elements and nuclear factors conferring Th2-specific expression of the IL-5 gene: a role for a GATA-binding protein. J. Immunol. *160*, 2343–2352.

Littlewood, T.D., Hancock, D.C., Danielian, P.S., Parker, M.G., and Evan, G.I. (1995). A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acid Res. *23*, 1686–1690.

Lu, B., Reichel, M., Fisher, D.A., Smith, J.F., and Rothman, P. (1997). Identification of a STAT6 domain required for IL-4-induced activation of transcription. J. Immunol. *159*, 1255–1264.

Mikita, T., Campbell, D., Wu, P., Williamson, K., and Schindler, U. (1996). Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. Mol. Cell. Biol. *16*, 5811–5820.

Moriggl, R., Berchtold, S., Friedrich, K., Standke, G.J., Kammer, W., Heim, M., Wissler, M., Stocklin, E., Gouilleux, F., and Groner, B. (1997). Comparison of the transactivation domains of Stat5 and Stat6 in lymphoid cells and mammary epithelial cells. Mol. Cell. Biol. *17*, 3663–3678.

Mosmann, T., and Coffman, R. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu. Rev. Immunol. *7*, 145–173.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A., and Coffman, R.L. (1986). Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. *136*, 2348–2357.

Murphy, K.M., Heimberger, A.B., and Loh, D.Y. (1990). Induction by antigen of interthymic apoptosis of $CD4^+$ $CD8^+$ TCR^{lo} thymocytes in vivo. Science *250*, 1720–1722.

Murphy, E., Shibuya, K., Hosken, N., Openshaw, P., Maino, V., Davis, K., Murphy, K., and O'Garra, A. (1996). Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. J. Exp. Med. *183*, 901–913.

Murphy, K.M., Ouyang, W., Szabo, S.J., Jacobson, N.G., Guler, M.L., Gorham, J.D., Gubler, U., and Murphy, T.L. (1999). T helper differentiation proceeds through Stat1-dependent, Stat4-dependent and Stat4-independent phases. Curr. Top. Microbiol. Immunol. *238*, 13–26.

Nakamura, T., Lee, R.K., Nam, S.Y., Podack, E.R., Bottomly, K., and Flavell, R.A. (1997). Roles of IL-4 and IFN-γ in stabilizing the T helper cell type 1 and 2 phenotype. J. Immunol. *158*, 2648–2653.

Nelms, K., Keegan, A.D., Zamorano, J., Ryan, J.J., and Paul, W.E. (1999). The IL-4 receptor: signaling mechanisms and biologic functions. Annu. Rev. Immunol. *17*, 701–738.

O'Garra, A. (1998). Cytokines induce the development of functionally heterogenous T helper cell substes. Immunity *8*, 275–283.

Onishi, M., Kinoshita, S., Morikawa, Y., Shibuya, A., Phillips, J., Lanier, L.L., Gorman, D.M., Nolan, G.P., Miyajima, A., and Kitamura, T. (1996). Applications of retrovirus-mediated expression cloning. Exp. Hematol. *24*, 324–329.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C., and Murphy, K.M. (1998). Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity *9*, 745–755.

Pritchard, C.A., Samuels, M.L., Bosch, E., and McMahon, M. (1995). Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. Mol. Cell. Biol. *15*, 6430–6442.

Quelle, F.W., Shimoda, K., Thierfelder, W., Fischer, C., Kim, A., Ruben, S.M., Cleveland, J.L., Pierce, J.H., Keegan, A.D., Nelms, K., et al. (1995). Cloning of murine Stat6 and human Stat6, Stat proteins that are tyrosine phosphorylated in responses to IL-4 and IL-3 but are not required for mitogenesis. Mol. Cell. Biol. *15*, 3336–3343.

Ranganath, S., Ouyang, W., Bhattarcharya, D., Sha, W.C., Grupe, A., Peltz, G., and Murphy, K.M. (1998). GATA-3-dependent enhancer activity in IL-4 gene regulation. J. Immunol. *161*, 3822–3826.

Robinson, D., Shibuya, K., Mui, A., Zonin, F., Murphy, E., Sana, T., Hartley, S.B., Menon, S., Kastelein, R., Bazan, F., and O'Garra, A. (1997). IGIF does not drive Th1 development but synergizes with IL-12 for interferon-γ production and activates IRAK and NFκB. Immunity *7*, 571–581.

Rogge, L., Barberis, M.L., Biffi, M., Passini, N., Presky, D.H., Gubler, U., and Sinigaglia, F. (1997). Selective expression of an interleukin-12 receptor component by human T helper 1 cells. J. Exp. Med. *185*, 825–831.

Romagnani, S. (1994). Lymphokine production by human T cells in disease states. Annu. Rev. Immunol. *12*, 227–257.

Ryan, J.J., McReynolds, L.J., Keegan, A., Wang, L.H., Garfein, E., Rothman, P., Nelms, K., and Paul, W.E. (1996). Growth and gene expression are predominantly controlled by distinct regions of the human IL-4 receptor. Immunity *4*, 123–132.

Shimoda, K., van, D.J., Sangster, M.Y., Sarawar, S.R., Carson, R.T., Tripp, R.A., Chu, C., Quelle, F.W., Nosaka, T., Vignali, D.A., et al. (1996). Lack of IL-4-induced Th2 response and IgE class switching in mice with disrupted Stat6 gene. Nature *380*, 630–633.

Stamatoyannopoulos, J.A., Goodwin, A., Joyce, T., and Lowrey, C.H. (1995). NF-E2 and GATA binding motifs are required for the formation of DNase I hypersensitive site 4 of the human β-globin locus control region. EMBO J. *14*, 106–116.

Swain, S.L., Weinberg, A.D., English, M., and Huston, G. (1990). IL-4 directs the development of Th2-like helper effectors. J. Immunol. *145*, 3796–3806.

Szabo, S.J., Jacobson, N.G., Dighe, A.S., Gubler, U., and Murphy, K. M. (1995). Developmental commitment in the Th2 lineage by extinction of IL-12 signaling. Immunity *2*, 665–675.

Szabo, S.J., Dighe, A.S., Gubler, U., and Murphy, K.M. (1997). Regulation of the interleukin (IL)-12Rβ2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J. Exp. Med. *185*, 817–824.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T., and Akira, S. (1996). Essential role of Stat6 in IL-4 signaling. Nature *380*, 627–630.

Takemoto, N., Koyano-Nakagawa, N., Yokota, T., Arai, N., Miyatake, S., and Arai, K.I. (1998). Th2-specific DNase I-hypersensitive sites in the murine IL-13 and IL-4 intergenic region. Int. Immunol. *10*, 1981–1985.

Wang, H.Y., Paul, W.E., and Keegan, A.D. (1996). IL-4 function can be transferred to the IL-2 receptor by tyrosine containing sequences found in the IL-4 receptor α chain. Immunity *4*, 113–121.

Woods, D., Parry, D., Chervinski, H., Bosch, E., Lees, E., and McMahon, M. (1997). Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by $p21^{Cip1}$. Mol. Cell. Biol. *17*, 5598–5611.

Zamorano, J., and Keegan, A.D. (1998). Regulation of apoptosis by tyrosine-containing domains of IL-4Rα: Y497 and Y713, but not the STAT6-docking tyrosines, signal protection from apoptosis. J. Immunol. *161*, 859–867.

Zhang, D.H., Cohn, L., Ray, P., Bottomly, K., and Ray, A. (1997). Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene. J. Biol. Chem. *272*, 21597–21603.

Zhang, D.H., Yang, L., and Ray, A. (1998). Differential responsiveness of the IL-5 and IL-4 genes to transcription factor GATA-3. J. Immunol. *161*, 3817–3821.

Zheng, W., and Flavell, R.A. (1997). The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell *89*, 587–596.